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Identification, Cloning, and Mutational Analysis of the Casein Kinase 1 cDNA of the Malaria Parasite, *Plasmodium falciparum*

STAGE-SPECIFIC EXPRESSION OF THE GENE*

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The cDNA for casein kinase 1 (CK1) of *Plasmodium falciparum* was cloned, sequenced, and expressed in bacteria. The single major open reading frame of the 1.2-kilobase pair cDNA coded for a 324-amino acid polypeptide of ~37 kDa, the predicted sequence of which showed strong identity with known CK1 isoforms. The purified recombinant enzyme exhibited properties characteristic of CK1, such as inhibition by CK1–7, the ability to phosphorylate a highly specific peptide substrate, and a strong preference for ATP over GTP. A casein kinase activity, partially purified from soluble extracts of *P. falciparum* by affinity chromatography through CK1–7 columns displayed identical properties. The activity showed a stage-specific expression in the parasite, in the order trophozoite > ring ≫ schizont. Northern analysis indicated the existence of two major CK1 mRNAs, 2.4 and 3.2 kilobase pairs long, the levels of which were in the order ring > schizont > trophozoite. Mutagenesis of recombinant CK1 defined important amino acid residues and their potential role in the conformation of the enzyme. The malarial CK1 appeared to be the one of the smallest and perhaps the most primitive CK1 enzymes known, containing little sequence information beyond the minimal catalytic domain.

The parasitic protozoan, *Plasmodium falciparum*, is the causative agent of malaria throughout the world and is responsible for an annual death toll of nearly 3 million, the majority of which are children and pregnant mothers (1). However, tools available to control malaria are inadequate, and drug-resistant strains are widespread; moreover, the immediate prospect of a useful vaccine is uncertain. Thus, there is an urgent need to obtain fundamental knowledge about the various cellular processes of *P. falciparum* at the molecular level, so that susceptible targets can be identified. With this long-term goal in mind, we have initiated studies of the signal transduction system in *P. falciparum*. Since reversible protein phosphorylation and dephosphorylation constitute a major mechanism of signal transduction (2), one of our immediate goals has been to characterize the various protein kinases in *P. falciparum*. In this paper, we report the characterization, expression, stage-specific regulation, and mutational analysis of *P. falciparum* casein kinase 1 (PfCK1).[1]

Casein kinase-1 and -2 (CK1 and CK2) are multipotential Ser/Thr protein kinases, originally purified from rabbit reticulocyte lysates using casein as substrate (reviewed in Refs. 3 and 4). In the subsequent years, both enzymes were shown to phosphorylate, and thus regulate, a wide variety of cellular proteins. The sequence alignment of CK1 genes of various organisms and deletion analysis of recombinant yeast CK1 have recently resulted in the delineation of the following domains in the prototype 45-kDa yeast enzyme (summarized in Refs. 5 and 6): an N-terminal catalytic domain of about 300 amino acids followed by a 12-residue stretch conserved among some forms but not in others; a hydrophilic 85-residue segment predicted to be flexible and rich in Pro and Ser; and finally, a 43-residue-long C-terminal prenylation site that contains the localization signal. The crystal structure of the 298-residue catalytic core of the *Schizosaccharomyces pombe* CK1 in complex with MgATP has recently been elucidated at 2.0-Å resolution (6). The structure suggested the roles of a number of conserved residues in various catalytic functions of the enzyme, such as binding of ATP, Mg^{2+}, and the phosphate groups of the substrate.

To confirm the identity of the malarial CK1 (PfCK1) cDNA, we have studied the biochemical properties of the recombinant malarial enzyme in detail and compared them with the native enzyme activity. In addition, preliminary mutational analysis of the enzyme was carried out to ascertain the essential nature of some of the invariant residues and their effect on the enzymatic parameters. Studies of stage-specific expression of the PfCK1 gene suggested transcriptional as well as post-transcriptional regulations. Our results constitute the first report of a CK1 enzyme in an eukaryotic protozoan parasite.

EXPERIMENTAL PROCEDURES

Parasite Culture and Extract—P. falciparum strain Dd2 was maintained in a modified erythrocyte culture (7, 8). Cultures were synchronized by purification of schizont stage over 65% Percoll (Pharmacia) cushion followed by incubation of ring stage parasites in 5% D-sorbitol (9). 1 liter of a synchronous *P. falciparum* culture of appropriate stage and at a parasitemia of 15–20% was treated with saponin (0.1% final concentration) to lyse erythrocytes. The parasite pellet was resuspended in the lysis buffer (50 mM Tris-Cl, pH 7.6, 2 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1% (v/v) aprotinin), homogenized, and briefly sonicated at 4 °C. The lysate was centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant (S100) was subjected to ammonium sulfate precipitation (0–60%) followed by resuspension and dialysis of the pellet fraction in the above buffer.

Isolation of *P. falciparum* genomic DNA and RNA from Saponin-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. A preliminary account of this work was presented at the Molecular Parasitology Meeting VII in Woods Hole, MA (September 15–19, 1996).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF017139.

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[1] The abbreviations used are: PfCK1, *P. falciparum* casein kinase 1; CK1 and CK2, casein kinase-1 and -2, respectively; kb, kilobase pair; PAGE, polyacrylamide gel electrophoresis.

lysed *P. falciparum*-infected erythrocytes and cloning of the cDNA into pBlueScript have been described (10).

Expression of the Malarial CK1 Gene in Bacteria—The putative PfCK1 gene open reading frame (975 base pairs) was subcloned from pBlueScript-SK± to the T7-based bacterial expression plasmid pET3a as follows. The gene was amplified by the following primers (corresponding to the 5′- and 3′-ends of the gene, respectively): 5′-GGAGTTGCATATGGAAATTAGAGTGGCA-3′ and 5′-GAGGATCCATCAATTATTTTCGTTGATCTC-3′ (the *Nde*I and *Bam*HI sites are underlined). The PCR product was restricted with *Nde*I and *Bam*HI and cloned into the same two sites of pET3a or pET15b as described (11). The resulting clones, designated pET3a-PfCK1 or pET15b-PfCK1, were confirmed by DNA sequencing and introduced into *E. coli* BL21(DE3). Growth of the transformant, induction with isopropyl-1-thio-β-D-galactopyranoside, and lysis with lysozyme-EDTA were carried out essentially as described previously (11). SDS-PAGE were performed according to Laemmli (12), using a 14% acrylamide (acrylamide:bisacrylamide = 30:0.4) gel for proteins and a 40% gel for peptides.

Site-directed mutagenesis and deletion of the cloned CK1 gene was performed by the PCR-based "megaprimer" method as described previously (13).

Purification of Recombinant PfCK1—The total extract of 1 g of induced BL21(DE3) cells containing the pET3a-PfCK1 plasmid was prepared as above and sonicated to shear the chromosomal DNA and reduce viscosity (5). The lysate was centrifuged at 120,000 × *g*, and the supernatant (S100) containing soluble recombinant CK1 was loaded on an 8-ml DEAE-cellulose column equilibrated with buffer A (50 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 5% glycerol, 1 mM dithiothreitol) containing 20 mM NaCl. Using a NaCl gradient in buffer A, the enzyme was subsequently eluted at an NaCl concentration of about 80 mM. The pooled fractions were directly applied to a 5-ml phosphocellulose column equilibrated with buffer A plus 80 mM NaCl and then eluted at about 0.5 M NaCl in a salt gradient. The phosphocellulose fraction, already highly pure (Fig. 8, *lane 1*), was concentrated by filtration through a Centricon-10 cartridge and further purified by a gel filtration chromatography through Sephadex G-50 (Fig. 8, *lane 2*), from which it eluted as an apparent monomer (data not shown).

Polyhistidine-tagged CK1 and its mutants (from the pET15b-PfCK1 clone) were purified essentially as described (5). For comparison, purified recombinant CK1δ subunit, expressed in *Escherichia coli* (14), was purchased from New England Biolabs (Bedford, MA).

Affinity Chromatography of CK1—Sepharose coupled to the isoquinolinesulfonamide compound CK1–7 (Seikagaku America, Ijaxville, MD), a specific CK1 inhibitor, was generated by reacting 5 mg of CK1–7 with 0.5 ml of CNBr-activated Sepharose 4B following standard procedures. Affinity chromatography of CK1 enzyme on this column was performed essentially as suggested (15), with some modifications as follows. 0.1 ml (1.0 mg of protein) of malarial S100 extract was passed four times through the CK1–7 column. The column was washed first with 1 ml of buffer B containing 1 mM dithiothreitol and then with 0.5 ml of 0.2 M arginine. The bound CK1 was eluted with 0.3 ml of 0.8 M arginine (15). The eluate was dialyzed overnight against buffer B containing 5 mM β-mercaptoethanol and stored frozen at −80 °C.

Casein Kinase Assay—Unless otherwise mentioned, standard protein kinase assay reactions (10 μl) contained the following: 20 μg α-casein (not dephosphorylated; from Sigma) or 1 mM of the synthetic peptide KRRRALpSVASLPGL (where pS represents phosphoserine) (New England Biolabs), 15 μM (10 μCi) [γ-^{32}P]ATP (or [γ-^{32}P]GTP of same specific activity, where mentioned), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50–100 ng of purified kinase in buffer B (50 mM Tris-Cl, pH 7.5, 120 mM NaCl, 5% glycerol). Where indicated, heparin or CK1–7 was also included in the reaction. 10 mM stock solutions of CK1–7 were made in Me_2SO, from which appropriate dilutions were made in water immediately before use. Reactions were initiated by the addition of the kinase and incubated for 15 min at 37 °C. When the synthetic peptide was the substrate, 2–4 μl of the reaction was stopped by the addition of SDS-sample buffer, boiled for 5 min, and analyzed by electrophoresis in 40% polyacrylamide gels containing SDS (16), followed by autoradiography. Where needed, the autoradiographs were scanned densitometrically. When casein was used as substrate, ^{32}P incorporation was quantitated by trichloroacetic acid precipitation on Whatman 31-ET paper (17). One unit of casein kinase was defined as the activity capable of incorporating 1 nmol of phosphate to casein in 1 h at 37 °C. The catalytic parameters (V_{max}, K_{cat}, and K_m) were measured under standard assay conditions and calculated by the Eadie-Hofstee method (18, 19). K_m of casein was calculated on the basis of its assumed molecular mass of 24 kDa. Casein kinase 2 (CK2) reactions were performed under identical conditions using recombinant CK2 (11).

RESULTS

Identification of the CK1 cDNA Homolog in the Malaria Parasite—Sequencing of random clones from a *P. falciparum* cDNA library in the Malaria Parasite Gene Sequencing Project (20) identified a clone with a 1.3-kb insert, which displayed significant homology to CK1 sequences in the National Center for Biotechnology Information data base. This putative CK1 gene, designated the PfCK1 gene, contained a 975-nucleotide-long open reading frame with the potential to code for a polypeptide of 324 amino acids. The 3′-untranslated sequence of PfCK1 was 190 nucleotides long, followed by a poly(A) tail. The deduced sequence of PfCK1 polypeptide showed extensive similarity with the N-terminal catalytic region of known CK1 polypeptides. A representative comparison with human and yeast CK1 is shown in Fig. 1. PfCK1 protein was found to be 59% similar to the human sequence; the similarity increased to 69% if conservative replacements were also included. Of particular significance was the finding that PfCK1 contained essentially all of the amino acid residues that were completely invariant in all known CK1 proteins (Fig. 1, *asterisks*). The seven PfCK1 residues that were exceptions to this are indicated by *dots above* them (Fig. 1); three of these were nonconservative replacements, *viz*. Thr^{78}, Tyr^{165}, and Ser^{245}, the corresponding invariant residues in all other CK1s being Asn, Ile, and Pro, respectively. Since the malarial CK1 was catalytically active (see below), we can conclude that these three residues may not be important in catalysis.

Organization of the PfCK1 Gene—To gather a preliminary knowledge of the PfCK1 gene organization, we carried out a Southern blot analysis of restriction endonuclease-digested *P. falciparum* genomic DNA using ^{32}P-labeled PfCK1 cDNA insert as probe. As shown in Fig. 2*A*, the majority of the restriction enzymes produced a single band, suggesting the existence of a single CK1 gene. RNA blot (Northern) analysis (Fig. 2*B*) revealed the existence of two size classes of transcripts, *viz*. 2.4 and 3.2 kb. Since the combined length of the PfCK1 coding sequence and the 3′-untranslated sequence is 1.17 kb, the 5′-untranslated sequence appears to be at least 1.2 kb in length. This rather long 5′-untranslated sequence is typical of many malaria parasite transcripts (10). It remains to be determined whether the two transcripts are due to different transcription events or are products of alternate splicing.

Characterization of CK1 Activity of the Malaria Parasite—To identify CK1 activity in *P. falciparum*, we subjected its S100 extract to affinity chromatography using the isoquinolinesulfonamide compound, CK1–7 (15). A malarial protein fraction obtained in this manner exhibited a potent casein kinase activity (Fig. 3) that was resistant to heparin (90% activity with 25 μg/ml heparin) but sensitive to CK1–7 (66 and 85% inhibition with 10 μM and 20 μM CK1–7, respectively).

The native malarial CK1 fraction also efficiently phosphorylated the CK1-specific peptide substrate, KRRRALpSVASLPGL, where the underlined Ser is the CK1 phosphorylation site (Fig. 4). 40 μM CK1–7 inhibited phosphorylation by nearly 80% at an ATP concentration of 15 μM (*lane 4*). The recombinant CK1δ isoform of rat testis expressed in *E. coli* (22) exhibited an essentially similar inhibition by CK1–7 (*lane 2*). Interestingly, heparin (10 μg/ml) stimulated the activity about 2-fold. As discussed later, the unique stimulatory effect of heparin, observed only when a small peptide was used as substrate, was also demonstrated with the CK1δ isoform (17). The enzyme demonstrated a marked preference for ATP; GTP was used much less efficiently (Fig. 4, *lane 6*). Together, these results demonstrate that *P. falciparum* contains a protein kinase activity essentially identical to CK1.

Stage-specific Expression of PfCK1 mRNA and Enzyme—The

```
                          •         •                           42
     PfCK1  ........ME IRVANKYALG KKLGSGSFGD IYVAKDIVTM EEFAVKLEST
  HumanCK1  MASSSGSKAE FIVGGKYKLV RKIGSGSFGD IYLAINITNG EEVAVKLESQ
 SpombeCK1  ......MTVD IKIGNKYRIG RKIGSGSFGQ IYLGLNTVNG EQVAVKLEPL

 Consensus  ---------E I-VGNKY-LG RKIGSGSFGD IYLA-NIVNG EEVAVKLES-
                          *         ** * **                * * *
                                    •             •             92
     PfCK1  RSKHPQLLYE SKLYKILGGG IGVPKVYWYG IEGDFTIMVL DLLGPSLEDL
  HumanCK1  KARHPQLLYE SKLYKILQGG VGIPHIRWYG QEKDYNVLVM DLLGPSLEDL
 SpombeCK1  KARHHQLEYE FRVYNILKGN IGIPTIRWFG VTNSYNAMVM DLLGPSLEDL

 Consensus  KARHPQLLYE SKLYKIL-GG IGIP-IRWYG -E-DYN-MVM DLLGPSLEDL
                  *  *    *         **     *      *      *********
                                                               142
     PfCK1  FTLCNRKFSL KTVRMTADQM LNRIEYVHSK NFIHRDIKPD NFLIGRGKKV
  HumanCK1  FNFCSRRFTM KTVLMLADQM ISRIEYHVTK NFIHRDIKPD NFLMGIGRHC
 SpombeCK1  FCYCGRKFTL KTVLLLADQL ISRIEYVHSK SFLHRDIKPD NFLM..KKHS

 Consensus  F--C-RKFTL KTVLMLADQM ISRIEYVHSK NFIHRDIKPD NFLMG-GKH-
               * * *   **    * *         *       ** *   ***
                               •    •                          192
     PfCK1  TLIHIIDFGL AKKYRDSRSH TSYPYKEGKN LTGTARYASI NTHLGIEQSR
  HumanCK1  NKLFLIDFGL AKKYRDNRTR QHIPYREDKN LTGTADYASI NAHLGIEQSR
 SpombeCK1  NVVTMIDFGL AKKYRDFKTH VHIPYRDNKN LTGTARYASI NTHIGIEQSR

 Consensus  N----IDFGL AKKYRD-RTH -HIPYRE-KN LTGTARYASI NTHLGIEQSR
                  ***   * * *  *    **    *    **    *    * * ****
                                                               242
     PfCK1  RDDIEALGYV LMYFLRGSLP WQGLKAISKK DKYDKIMEKK ISTSVEVLCR
  HumanCK1  RDDMESLGYV LMYFNRTSLP WQGLKAATKK QKYEKISEKK MSTPVEVLCK
 SpombeCK1  RDDLESLGYV LLYFCRGSLP WQGLQADTKE QKYQRIRDTK IGTPLEVLCK

 Consensus  RDD-ESLGYV LMYF-RGSLP WQGLKA-TKK QKY-KI-EKK ISTPVEVLCK
            *** *  *           ** **** *          *   *        *
              •                                                292
     PfCK1  NASFEFVTYL NYCRSLRFED RPDYTYLRRL LKDLFIREGF TYDFLFDWTC
  HumanCK1  GFPAEFAMYL NYCRGLRFEE APDYMYLRQL FRILFRTLNH QYDYTFDWTM
 SpombeCK1  GLPEEFITYM CYTRQLSFTE KPNYAYLRKL FRDLLIRKGY QYDYVFDWMI

 Consensus  G-P-EF-TYL NYCR-LRFEE -PDY-YLR-L FRDLFIR-G- QYDY-FDWT-
              *     *       * *    * *  *                  *   **
                                        324
     PfCK1  VYASEKDKKK MLENKNRFDQ TADQEGRDQR NN........ ..........
  HumanCK1  LK..QKAAQQ AASSSGQGQQ AQTPTGKQTD KTKSNMKGF. ..........
 SpombeCK1  LKYQKRAAAA AAASATAPPQ VTSPMVSQTQ PVNPITPNYS SIPLPAERNP

 Consensus  LK---KAA-- AA-S-----Q ---P-G-QT- ---------- ----------


 SpombeCK1  KTPQSFSTNI VQCASPSPLP LSFRSPVPNK DYEYIPSSLQ PQYSAQLRRV

 SpombeCK1  LDEEPAP
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FIG. 1. **Amino acid sequence alignment of CK1 polypeptides.** The predicted amino acid sequences of the malarial, human (21), and yeast (*S. pombe*; Ref. 22) CK1 polypeptides have been compared using the PILEUP and PRETTY programs of the Genetics Computer Group package (Madison, WI). The *numberings* are based on the malarial sequence. *Asterisks* denote amino acid residues that are invariant in all known CK1 isoforms identified to date; the malarial residues that are exceptions to this rule are indicated by *dots above* the *residues*.

malaria parasite undergoes distinct morphological changes during the progression through its intraerythrocytic life cycle: rings (initial maturation stage following invasion), trophozoites (larger mononucleated metabolically active cells containing hemozoin pigment), and schizonts (multinucleated cells). To address whether the PfCK1 gene is expressed in a stage-specific manner through this intricate life cycle, RNA samples were isolated from synchronized cultures every 6 h. As shown by Fig. 5, the expression of PfCK1 transcripts is indeed cell cycle-regulated. Both size classes of transcript showed a peak expression in the early ring stage and were barely detectable in trophozoite and schizont stages.

Interestingly, when we determined the specific activity of CK1 in the S100 extracts of the various stages of the parasite, a different picture emerged. Using the specific phosphopeptide substrate, the highest CK1 activity was found in trophozoites, followed by rings, while the activity in the schizonts was barely detectable (Fig. 6). The relative activities in the three stages, determined by densitometric scanning, were in the ratio 100: 42:3. The authenticity of the CK1 activity was further verified by its sensitivity to the inhibitor CK1–7 and by comparison to CK1δ (Fig. 6). At 20 μM CK1–7, for example, activities of all stages were inhibited by 85–90%.

Endogenous Substrates of Malarial CK1—In an attempt to obtain a preliminary estimate of the natural substrates of the malarial CK1, we subjected the S100 extracts of the three major stages of the parasite to self-phosphorylation, whereby equal amounts of the S100 proteins were incubated in the presence of [γ-^{32}P]ATP and increasing concentrations of CK1–7. Analysis of the reaction products on SDS-PAGE, as shown in Fig. 7, led to a number of findings, summarized as follows. (*a*) A number of malarial polypeptides appeared to serve as substrates for endogenous kinases. The major phosphoprotein species common in both rings and trophozoites exhibited molecular masses of 66, 60, 33, and 27 kDa in addition to a number of high molecular weight species, whereas the 42-

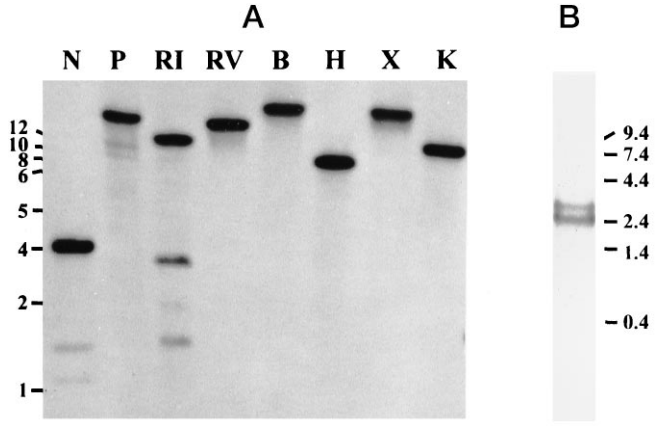

FIG. 2. *A*, Southern analysis of *P. falciparum* Dd2 genomic DNA. 3 μg of genomic DNA, restricted with the following enzymes, was subjected to Southern analysis using PfCK1 cDNA as probe (10). *Lane 1*, *Nde*I; *lane 2*, *Pst*I; *lane 3*, *Eco*RI; *lane 4*, *Eco*RV; *lane 5*, *Bam*HI; *lane 6*, *Hin*dIII; *lane 7*, *Xba*I; *lane 8*, *Kpn*I. *B*, Northern analysis of CK1 RNA. 10 μg of total *P. falciparum* RNA from asynchronous culture was probed with PfCK1 cDNA (10). Standard DNA and RNA markers are shown.

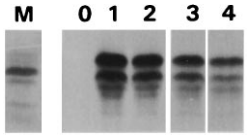

FIG. 3. **Casein kinase activity of native PfCK1.** Approximately 20 ng of malarial enzyme fraction purified by affinity chromatography through a CK1–7 column was tested for casein kinase activity in standard phosphorylation reactions, followed by analysis in SDS-PAGE. *Lane M* represents a stained lane containing casein; others lanes are autoradiographs. Phosphorylation reactions were carried out with the following additions: none (*lane 1*); 25 μg/ml heparin (*lane 2*); 10 μM CK1–7 (*lane 3*); and 20 μM CK1–7 (*lane 4*). *Lane 0* is a control (no enzyme).

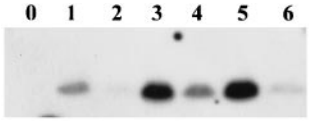

FIG. 4. **Activity of native PfCK1 on a specific peptide substrate.** Phosphorylation of the peptide KRRRALpSVASLPGL by 20 ng of native (CK1–7 affinity-purified) malarial CK1 (*lanes 3–6*) or by commercial CK1 (*lanes 1* and *2*) and analysis of the reactions by SDS-PAGE followed by autoradiography were carried out as described under "Experimental Procedures." Phosphate donors were [γ-^{32}P]ATP (lanes 0–5) or [γ-^{32}P]GTP (*lane 6*). The additions to the phosphorylation reactions were as follows: none (*lanes 1*, *3*, and *6*); 40 μM CK1–7 (*lanes 2* and *4*); 10 μg/ml heparin (*lane 5*).

and 36-kDa species were predominant in rings only. The schizonts, on the other hand, exhibited a pattern that is very different from either rings or trophozoites and consisted of two major species of about 98 and 75 kDa. In all three extracts, most of the phosphoproteins appeared to be minor *Plasmodium* S100 proteins, as judged by comparing the bands in the autoradiogram with those in the corresponding Coomassie Blue-stained gel (*panel A*). (*b*) With increasing concentrations of CK1–7, phosphorylated bands of both ring and trophozoites started to disappear much earlier than those of the schizont. At 50 μM CK1–7, for example, phosphorylation of ring and trophozoite extracts was almost completely inhibited, whereas that of schizonts remained substantially unaffected. Together, these results suggest that CK1 may be a major protein kinase in the ring and trophozoite stages, while the schizont stage may have a different kinase make-up. (*c*) In an effort to assess whether the stage-specific phosphoprotein pattern was due to a difference in the level of substrates or enzymes, purified recombi-

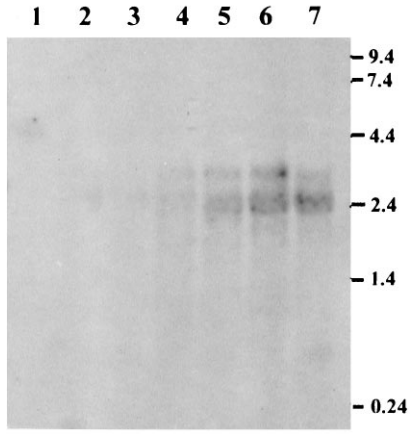

FIG. 5. **Stage-specific expression of the PfCK1 mRNA.** Total RNA was isolated every 6 h from a synchronous *P. falciparum* Dd2 culture. Approximately 4 μg of RNA sample from each time point was analyzed as in Fig. 2*B*. *Lane 1*, early trophozoite; *lane 2*, trophozoite; *lane 3*, mature trophozoite/early schizont; *lane 4*, schizont; *lane 5*, segmented; *lane 6*, early ring; *lane 7*, ring. Positions of standard RNA markers (in kb) are shown. The relative amounts of the transcripts, determined by densitometric scanning of the autoradiograph, are in the following ratio (*left* to *right*): 4:14:12:22:45:100:98.

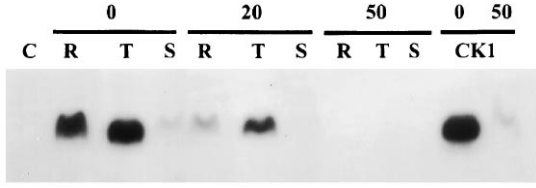

FIG. 6. **Stage-specific PfCK1 activity.** Peptide phosphorylation assays were carried out exactly as described in the Fig. 4 legend using 4 μg of stage-specific extracts (ring (*R*); trophozoite (*T*); schizont (*S*)), prepared as described under "Experimental Procedures." *Numbers* on the *top* indicate CK1–7 concentrations (μM) used in the reaction. *Lane C* is a reaction without any kinase; *lanes* marked *CK1* represent reactions with commercial CK1δ.

nant malarial CK1 was added to extracts of all three stages (Fig. 7, *lanes with plus sign*). Interestingly, this restored the schizont pattern to resemble those of ring and trophozoite, while the patterns of the latter two stages remained virtually unaltered. As expected, only those bands in the schizont extracts that were due to the exogenously added CK1 exhibited sensitivity to CK1–7. These results strongly suggest that schizonts are substantially devoid of CK1 activity rather than CK1 substrates.

Enzymatic Activity of Bacterially Expressed Malarial CK1—Upon induction of BL21(DE3) containing pET3a-PfCK1 with isopropyl-1-thio-β-D-galactopyranoside, a polypeptide of M_r ~37,000 was produced (Fig. 8), which is in reasonable agreement with the predicted molecular weight of 37,807 for the CK1 open reading frame polypeptide, considering the slight overall basicity of the predicted polypeptide (42 Asp and Glu residues; 55 Lys and Arg residues). The 37-kDa protein was purified by chromatography through DEAE and phosphocellulose and then by gel filtration using Sephadex G50. During purification, the casein kinase activity always co-chromatographed with the 37-kDa polypeptide. The final preparation (Fig. 8, *lane 2*) was at least 90% pure, with no single contaminant constituting more than 5% of the 37-kDa polypeptide. *In vitro*, the purified recombinant enzyme efficiently phosphorylated casein (Fig. 9) with a specific activity of ~1020 units/μg of protein. The activity was resistant to heparin (25 μg/ml) but was inhibited by about 70 and 80% in the presence of 10 and 20 μM CK1–7, respectively (Fig. 9). Under the same reaction conditions, purified CK2 was inhibited by heparin but retained about 90 and

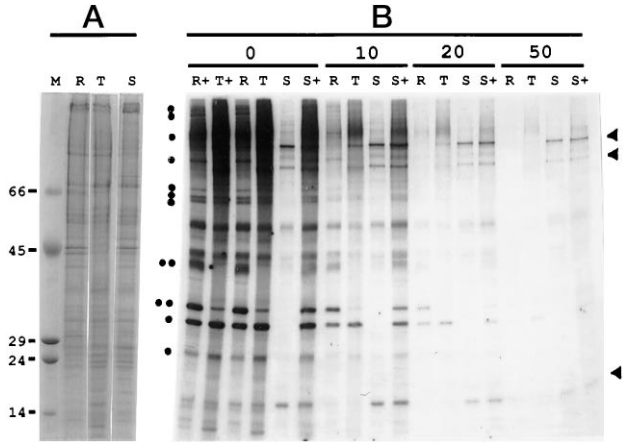

FIG. 7. **Endogenous phosphorylation of *P. falciparum* extracts.** 5 μg of cytosolic extracts of ring (*R*), trophozoite (*T*), and schizont (*S*) stages of *Plasmodium* were self-phosphorylated in a standard kinase reaction using [γ-^{32}P]ATP without any exogenous substrate. The reactions were analyzed in SDS-PAGE and autoradiographed (*panel B*). *Panel A* represents stained gel containing 80 μg of each extract as indicated. *Numbers* on the *left* indicate sizes of protein markers (kDa); *numbers* on *top* indicate CK1–7 concentrations (μM) included in the reaction. The *plus signs* indicate reactions in which an additional 50 ng of purified recombinant PfCK1 was added to the extracts. Major phosphoprotein bands are marked as follows according to their presence in the extracts: both ring and trophozoites (*single dots*); rings only (*double dots*); schizont-specific (*arrowheads*).

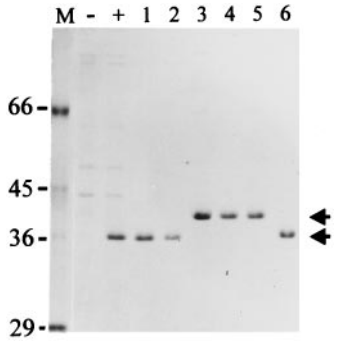

FIG. 8. **Expression of recombinant PfCK1 in *E. coli*.** Malarial CK1 or its His fusion derivatives were cloned and expressed in pET3a or pET15b, respectively, as described under "Experimental Procedures." BL21(DE3) cells containing the following recombinant CK1 clones were induced with isopropyl-1-thio-β-D-galactopyranoside (− *lane*; or uninduced, + *lane*), and total extracts (− and + *lanes*) or purified fractions (*lane 1*, phosphocellulose fraction; *lane 2*, Sephadex G-50 fraction) were analyzed on SDS-PAGE followed by staining with Coomassie Blue: wild type CK1 (*lanes* +, −, *1*, and *2*); purified poly-His-tagged wild type (*lane 3*); G21A mutant (*lane 4*); K38L mutant (*lane 5*), and the C-terminal Δ18 mutant (*lane 6*; see text for details). *Lane M* represents protein standards in kDa. The CK1 bands are indicated by *arrowheads*.

70% activity in the presence of 10 and 20 μM CK1–7, respectively (3, 14). Thus, the recombinant malarial CK1 fraction exhibited properties characteristic of the authentic CK1 class of enzymes. The catalytic parameters, measured in the presence of 15 μM ATP and 2 μg casein per μl were as follows: V_{max}, 17.5 ± 1.5 μmol of casein/mg of enzyme/min; K_{cat}, 15.5 ± 1.2 s^{-1}; K_m (casein), 110 ± 10 μM, producing a K_{cat}:K_m ratio of 8.45 μM^{-1} min^{-1}.

In an attempt to simplify the purification procedure and to be able to rapidly screen various mutants of CK1 in the future, we have also cloned PfCK1 into pET15b. This resulted in the fusion of the 2-kDa histidine-rich peptide $MGSS(H)_6SSGLVPRGSH$ to CK1, which increased the M_r of the recombinant protein to about 39 kDa. The fusion protein could be purified to near homogeneity in a single step using a

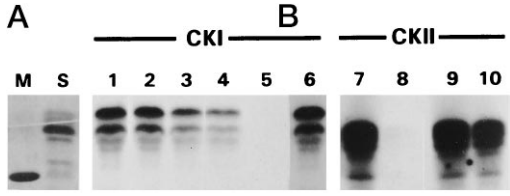

FIG. 9. **Phosphorylation of casein by recombinant PfCK1.** Casein was phosphorylated by purified recombinant malarial CK1 (*CKI*) or by control CK2 (*CKII*) in standard phosphorylation reactions using [γ-^{32}P]ATP as phosphate donor. The reactions were analyzed by SDS-PAGE, followed by staining and autoradiography. *Panel B* shows the autoradiograph, and *panel A* shows a representative stained portion of the gel (*M*, 20-kDa standard; *S*, casein). Phosphorylation reactions were carried out in the presence of the following: no additions (*lanes 1* and *7*); 25 μg/ml heparin (*lanes 2* and *8*); CK1–7, 10 μM (*lanes 3* and *9*), and 20 μM (*lanes 4* and *10*). *Lane 6* represents a reaction using commercial CK1 (New England BioLabs), and *lane 5* shows a control (no enzyme).

nickel column (Fig. 8, *lane 3*), and exhibited a specific activity essentially identical to that of the nonfusion recombinant (data not shown).

Mutational Analysis of Recombinant Malarial CK1—The peptide stretch G*X*G*XX*G (residues 16–21), followed by a Lys^{38} 16 residues downstream, is conserved in all CK1 catalytic cores and is considered to be involved in ATP binding (23). Crystallographic studies have suggested that the triphosphate moiety of ATP interacts with three charged residues of CK1, *viz*., Lys^{38}, Lys^{130}, and Asp^{151} and with the amide backbone of Gly^{21} (6). In addition, Asn^{133} has been implicated in the coordination of the single Mg^{+2} ion in CK1. However, no detailed mutational studies to validate these predictions have been performed. In an earlier report, the K38N mutant was reported to be "kinase-dead," although the exact mechanism of the defect was not studied (14). Since the malarial recombinant CK1 was enzymatically active, it gave us an opportunity to analyze the roles of specific residues by site-directed mutagenesis. Our initial studies employed His fusion PfCK1 mutants G21A and K38L expressed in pET-15b (Fig. 8, *lanes 4* and *5*). In standard casein kinase assays, both mutants were found to be highly defective. For detailed kinetic studies, however, we expressed these and other mutants in the pET-3a vector, free of any exogenous sequences. Kinetic data presented in Table I revealed that the K38L, K130L, and D151N mutants all had a highly elevated K_m for ATP and similarly defective K_{cat}. Interestingly, these mutants also exhibited a higher K_m for Mg^{2+}, which supports the notion that Mg^{2+} binds ATP, and that the Mg^{2+}-ATP complex is coordinated to the relevant residues of CK1. This is further confirmed by the reciprocal mutation N133A, which not only increased the K_m for Mg^{2+} as expected but also had a severe effect on the K_m for ATP. Substitution of Gly^{21} with alanine had the least effect on enzymatic parameters, probably because the two amino acids are very similar and the peptide bond of Gly^{21} rather than its side chain is postulated to interact with ATP (6).

The minimal length of a functional CK1 enzyme remains undefined. Truncation of the C-terminal 148 amino acids of the recombinant CKi1 of *S. pombe* (product of the *cki1* gene) generated a 298-residue core that contained only 3 residues beyond the invariant DW dipeptide (5). This "catalytic core" was enzymatically active and, as mentioned earlier, led to the only crystal structure of CK1 available to date (6). However, larger deletions of this or any other isoform of CK1 have not been reported. To delineate the minimal length of the CK1 catalytic core, we have carried out terminal deletions of the malarial enzyme in the recombinant pET-3a clone. The truncated mutants were expressed in bacteria, purified, and assayed using casein as substrate. Deletion of 18 amino acid residues from the

TABLE I
Kinetic parameters of mutant PfCK1 enzymes

Kinase reactions using casein as substrate and the determination of K_m and K_{cat} were carried out as described under "Experimental Procedures." All CK1 enzymes used in this experiment were expressed from the pET-3a vector and were devoid of any exogenous sequence, *viz* the His_6 fusion peptide. ΔC18 had near normal activity and, therefore, was not studied in detail. S.D. values were calculated from three independent measurements.

Enzyme	K_m of Mg^{2+}	K_m of ATP	K_{cat}
	μM	*μM*	$s^{-1} \times 100$
Wild type	12 ± 1	14 ± 1.5	1550 ± 170
K38L	1450 ± 130	2240 ± 150	12 ± 2
K130L	1235 ± 120	2500 ± 210	8 ± 1
D151N	1125 ± 90	1250 ± 110	10 ± 2
N133A	2250 ± 180	1125 ± 102	11 ± 2
G21A	15 ± 1.8	150 ± 12	72 ± 10
ΔC36	21 ± 2	25 ± 2	124 ± 11
ΔN8	32 ± 2	40 ± 3	115 ± 10

C terminus (ΔC18) of PfCK1 was found to retain 80% of the wild type activity. Essentially similar results were obtained when the ΔC18 mutant (Fig. 8, *lane 6*) had a His fusion at the N terminus. However, deletion of 36 residues from the C terminus, which resulted in the loss of the invariant DW, produced an essentially inactive enzyme. Deletion of only 8 residues from the N terminus also resulted in loss of activity, although an invariant Gly in this region is replaced with an Ala in PfCK1 (Fig. 1). Kinetic parameters of these two truncated enzymes, *viz*. ΔN8 and ΔC36, revealed an appreciable defect in their interaction with Mg-ATP and a greater defect in K_{cat} (Table I), thus defining the boundaries of a minimal CK1 catalytic core and confirming that the CKi1Δ298 of *S. pombe* was indeed close to this limit (5, 6).

DISCUSSION

In this paper, we have identified the PfCK1 gene and enzyme in the malaria parasite *P. falciparum*. We have also confirmed the roles of some of the conserved residues of PfCK1 through site-directed mutational studies and analyzed the parasite stage-specific expression of PfCK1 transcripts and its enzymatic activity. The native as well as the recombinant enzyme synthesized in *E. coli* showed properties diagnostic of CK1. Deletion analysis of PfCK1 (Table I) strongly suggested a role of the terminal regions outside the conserved catalytic core in modulating enzyme activity. Curiously, in the crystal structure, neither of these regions was predicted to be directly involved in either Mg-ATP binding or catalytic function (6). Thus, we presume that these terminal sequences may somehow influence the overall conformation of the enzyme or its active site. Nevertheless, these deletions helped us define the boundaries of the minimal catalytic core of CK1. To our knowledge, the 324-amino acid-long malarial CK1 enzyme reported here is one of the shortest naturally occurring CK1 and contains only 34 residues following the invariant DW dipeptide (Fig. 1). In this regard, it is comparable with, albeit slightly smaller than, the CK1 α and β isoforms of higher eukaryotes, the shortest of which is bovine CK1α, containing 325 amino acids (24). This probably underscores the more primitive evolutionary status of this obligatory parasite compared with its eukaryotic hosts. On a similar note, PPλ, a prokaryotic protein phosphatase, was recently been shown to contain very little sequence information beyond the conserved domains and, thus, appeared to be equivalent to the minimal catalytic core of the larger eukaryotic phosphatases (16).

Primarily through the analysis of deletion mutants, the C-terminal tail beyond the conserved region of CK1 has been postulated to be involved in a number of regulatory mechanisms, *viz.* autophosphorylation, autoinhibition, membrane localization, and heparin-mediated activation (Fig. 4) of CK1 activity, some of which may be interrelated (5, 17). In this respect, the PfCK1 enzyme also appears to be unique. First, it is in fact shorter than all CK1α isoforms and is still activated by heparin. Second, the short C terminus of PfCK1 has very little homology with other CK1 isoforms (Fig. 1). Third, in preliminary studies, PfCK1 did not exhibit any appreciable autophosphorylation (data not shown), suggesting that this may not be required for heparin activation of PfCK1. Finally, we did not get any C-terminal deletions of PfCK1 that exhibited higher activity than the full-length enzyme. The shortest deletion tested (ΔC18) actually lost about 20% activity but at the same time also lost heparin activation (data not shown). Thus, it appears that the malarial enzyme is unique in that it lacks autophosphorylation and autoinhibition but still retains heparin activation, which most likely maps within the last 18 residues of the C terminus. Perhaps heparin-activation requires a certain conformation in the C terminus that is attained through phosphorylation in other CK1 isoforms but is constitutively present in PfCK1. The significance of the potential regulation of PfCK1 by heparin in the parasitic life cycle awaits further study. Since the C-terminal region has been implicated in targeting the enzyme to the membranes (25), it will be interesting to determine whether malarial CK1 is primarily localized in the cytosolic compartment of the parasitic cell.

CK1 enzymes in general are known to require acidic residues (Asp or Glu) on the N-terminal side of the phosphorylatable Ser in the substrate, especially in the *n*-3 position, where the Ser is at position *n*; however, a phosphorylated residue at *n*-3 results in a pronouncedly higher K_{cat}:K_m ratio (26, 27). With either recombinant or native PfCK1, we have also seen pronouncedly better phosphorylation of the peptide substrate KRRRALpS-VASLPGL than the nonphosphorylated D4 peptide (Promega). This prompted us to use native casein that has not been dephosphorylated as the substrate for the CK1 assay *in vitro*. In our SDS-PAGE analysis, commercial casein migrated in three bands: a faster, major band and two minor, slower migrating ones. Malarial CK1 phosphorylated the slowest migrating band much more efficiently (Figs. 3 and 9), while CK2 preferentially phosphorylated the faster migrating one (Fig. 9). Commercially obtained recombinant CK1δ also resembled malarial CK1 in this respect (data not shown). Although we do not know the exact reason behind it, we speculate that the slower species may represent the more highly phosphorylated forms of casein and, thus, function as better substrates for CK1.

Several characteristics of the PfCK1 described here are reminiscent of a protein kinase activity previously described in partially purified cytosolic fractions of *Plasmodium* (28–30). In brief, the activity phosphorylated casein as well as phosvitin, required Mg^{2+}, preferred ATP over GTP, was stimulated by polyamines spermine and spermidine, and was inhibited by the flavone, quercitin. It also appeared that trophozoites possessed higher amounts of this activity than either the ring or schizont stages (30), which is similar to the stage-specific variation of PfCK1 activity that we have observed (Figs. 6 and 7). What is intriguing, however, is the apparent lack of correlation of the relative steady state levels of PfCK1 mRNA (Fig. 5) and enzyme activities (Fig. 6) in the various stages of *Plasmodium*. The schizonts, for example, have at least twice as much CK1 mRNA as the trophozoites; however, the schizonts possessed negligible amounts of CK1 activity, whereas the trophozoites exhibited the highest activity of all three stages. It is thus tempting to speculate that the *Plasmodium* CK1 expression undergoes stage-specific regulation at transcriptional as well as post-transcriptional levels. A distinction between transla-

tional and post-translation regulations will ideally require the use of a specific anti-PfCK1 antibody, which is currently unavailable.

The CK1 family of enzymes phosphorylates a variety of physiological substrates including transcription factor CREM (31), glycogen synthase (32), p53 (33), and the SV40 T antigen (34). Individual CK1 isoforms in lower eukaryotes are found in the cytoplasm as well as in the nuclei and play essential roles in the regulation of cellular morphogenesis and DNA repair (35–37). Self-phosphorylation of malarial cytoplasmic extracts revealed a number of phosphoproteins (Refs. 29 and 30; Fig. 7) of unknown identity. The unique phosphoprotein profile in the schizont stage (Fig. 7) was most easily explained by the exceedingly low CK1 activity in this stage compared with the others (Fig. 6) and by the fact that the addition of exogenous CK1 made the schizont profile resemble those of the other two stages (Fig. 7). However, the possibility that some substrate proteins themselves may also undergo stage-specific expression has been suggested (30) and merits further investigation for its obvious regulatory implications. In recent years, *Plasmodium* infection of human red blood cells was shown to result in elevated phosphorylation of a number of human red blood cell membrane proteins (38–41). This included protein 4.1, a 80-kDa polypeptide, the phosphorylation of which was enhanced in the trophozoite-schizont stage of infection, and appeared to involve a casein kinase activity (40). Although additional studies are needed to ascertain the exact nature of the kinase(s), this result raises the exciting possibility that the malarial CK1 may in fact modulate host-parasite interactions, which will have important consequences in the prevention and management of malaria. In any case, the demonstration of the CK1 gene and enzyme in *P. falciparum* and their regulation in the various stages of the protozoan life cycle should open up new directions of research in the signal transduction pathways of this clinically important parasite.

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Identification, Cloning, and Mutational Analysis of the Casein Kinase 1 cDNA of the Malaria Parasite, *Plasmodium falciparum* : STAGE-SPECIFIC EXPRESSION OF THE GENE

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